Chlorophyll and Chlorophyll Catabolite Analysis by HPLC

Das, Aditi ; Guyer, Luzia ; Hörtensteiner, Stefan

Abstract: The most obvious event of leaf senescence is the loss of chlorophyll. Chlorophyll degradation proceeds in a well-characterized pathway that, although being common to higher plants, yields a species-specific set of chlorophyll catabolites, termed phyllobilins. Analysis of chlorophyll degradation and phyllobilin accumulation by high-performance liquid chromatography (HPLC) is a valuable tool to investigate senescence processes in plants. In this chapter, methods for the extraction, separation, and quantification of chlorophyll and its degradation products are described. Because of their different physicochemical properties, chlorin-type pigments (chlorophylls and magnesium-free pheo-pigments) and phyllobilins (linear tetrapyrroles) are analyzed separately. Specific spectral properties and polarity differences allow the identification of the different classes of known chlorins and phyllobilins. The methods provided facilitate the analysis of chlorophyll degradation and the identification of chlorophyll catabolites in a wide range of plant species, in different tissues, and under a variety of physiological conditions that involve loss of chlorophyll.

DOI: https://doi.org/10.1007/978-1-4939-7672-0_18

Chlorophyll and chlorophyll catabolite analysis by HPLC

Aditi Das[1], Luzia Guyer[1] and Stefan Hörtensteiner*

Institute of Plant and Microbial Biology, University of Zurich, Zollikerstrasse 107, CH-8008 Zurich, Switzerland

[1] These authors contributed equally to this work

*Corresponding author: shorten@botinst.uzh.ch

Running title: Chlorophyll catabolite analysis

Abstract

The most obvious event of leaf senescence is the loss of chlorophyll. Chlorophyll degradation proceeds in a well-characterized pathway that, although being common to higher plants, yields a species-specific set of chlorophyll catabolites, termed phyllobilins. Analysis of chlorophyll degradation and phyllobilin accumulation by high performance liquid chromatography (HPLC) is a valuable tool to investigate senescence processes in plants. In this chapter, methods for the extraction, separation and quantification of chlorophyll and its degradation products are described. Because of their different physicochemical properties, chlorin-type pigments (chlorophylls and magnesium-free pheo-pigments) and phyllobilins (linear tetrapyrroles) are analyzed separately. Specific spectral properties and polarity differences allow the identification of the different classes of known chlorins and phyllobilins. The methods provided facilitate the analysis of chlorophyll degradation and the identification of chlorophyll catabolites in a wide range of plant species, in different tissues, and under a variety of physiological conditions that involve loss of chlorophyll.

Key words: Chlorophyll, pheophytin, pheophorbide, phyllobilin, leaf senescence, fruit ripening, HPLC, chlorophyll catabolite

1. Introduction

Chlorophyll, the most abundant plant pigment, absorbs sun light as a key factor for the conversion of solar energy to chemical energy during photosynthesis. At the same time, however, chlorophyll is a potentially phototoxic molecule, whose biosynthesis and degradation has to be tightly regulated. During leaf senescence, when photosynthetic activities diminish and photosystem protein components are degraded for nitrogen remobilization to other parts of the plant, chlorophyll is metabolized for its detoxification. Breakdown of chlorophyll has been used as a diagnostic tool not only for induction and progression of leaf senescence, but also for monitoring other processes of plant development, such as fruit ripening and seed maturation, and as plants' responses to biotic and abiotic stress challenges.

In recent years, a pathway of chlorophyll breakdown has been elucidated that converts chlorophylls, green-colored tetrapyrroles with a chlorin ring structure, to colorless, linear tetrapyrroles termed phyllobilins *(1)*. This pathway consists of several enzymatic steps that produce different short-lived catabolite intermediates and a plant species-specific set of phyllobilins that ultimately accumulate in the vacuole of senescing cells *(1, 2)*. The series of intermediary and final catabolites are depicted in **Fig. 1**. The pathway is structured into two parts: (i) reactions from chlorophyll to a "primary" fluorescent chlorophyll catabolite (*p*FCC) that are localized in the chloroplast and commonly occur in all plant species investigated so far *(3)*, and (ii) species-specific, largely cytosolic, reactions that site-specifically modify peripheral positions of *p*FCC and give rise to the more or less complex pattern of phyllobilins detectable in a given species *(4, 5, 6)*. The majority of phyllobilins that accumulate are nonfluorescent, because fluorescent precursors rather quickly isomerize to their nonfluorescent equivalents within the vacuole due the low pH of the vacuolar sap *(5, 7)*. Exceptions are so-called "hypermodified" FCCs found in a few species, in which modification of the propionyl side chain (**Fig. 1**) prevents efficient isomerization to their respective nonfluorescent equivalents *(8, 9)*. Two major classes of phyllobilins are distinguished, that are, formyloxobilin-type (FCCs and NCCs) and dioxobilin-type tetrapyrroles (DFCCs and DNCCs) (**Fig. 1**) *(6)*, the latter of which are (formally) derived from the former ones through an oxidative deformylation *(5)*. In some cases, NCCs were shown to be oxidized to yellow chlorophyll catabolites *(10)*.

Along the pathway of degradation, polarity of chlorophyll catabolites increases together with a general shift of absorption towards shorter wavelengths *(2, 6)*. These features allow (i) the separation of chlorophyll catabolites by reversed-phase HPLC, (ii) the identification of different groups of catabolites based on their characteristic UV/Vis absorption spectra (**Fig. 1**) and (iii) the quantification of several groups of compounds for which standards are available or can be relatively easily produced.

In this chapter, two methods for separation, identification and quantification of either chlorin- or phyllobilin-structured chlorophyll catabolites are described.

Figure 1 near here.

2. Materials

All chemicals used should be of analytical grade.

2.1. General solutions

1. Tris-HCl, pH 8.0.

2. 0.5 M KH_2PO_4.

3. 0.5 M K_2HPO_4.

4. 10x concentrated (0.5 M) KPi buffer: Mix solutions of 0.5 M K_2HPO_4 and 0.5 M KH_2PO_4, until reaching a pH of 7.0. 100 mL of 0.5 M KPi buffer requires approximately 42 mL of 0.5 M K_2HPO_4 and 58 mL of 0.5 M KH_2PO_4. Filter through a 0.45 µm nylon filter.

5. 1 M ammonium acetate: Filter through a 0.45 µm nylon filter, store at 4° C.

6. Acetone.

7. Methanol (MeOH): HPLC grade.

8. Milli-Q water (18 MΩ).

2.2. Chlorophyll/phyllobilin extraction

1. Chlorin extraction buffer: 0.2 M Tris-HCl, pH 8.0, acetone, 10:90 (v/v) (*see* **Note 1**).

2. Phyllobilin extraction buffer: 50 mM potassium phosphate (KPi) buffer, pH 7.0, methanol, 25:75 (v/v) (*see* **Note 2**).

3. Liquid nitrogen.

4. Mortars and pestles (*see* **Note 3**).

5. Microcentrifuge tubes: 1.5 and 2 mL.

6. Holders for microcentrifuge tubes.

7. Spatula and thumb forceps.

8. Analytical balance.

9. Microcentrifuge.

10. Vortex mixer.

11. Pipettes and pipette tips.

12. Conical HPLC vials (200-300 µL) and caps.

2.3. HPLC analysis

2.3.1. HPLC analysis of chlorins (*see* **Note 4**)

1. Solvent A: Mix 1 M ammonium acetate and MeOH with the ratio 20:80 (v/v) (*see* **Note 5**).

2. Solvent B: Mix acetone and MeOH with the ratio 20:80 (v/v).

3. C18 Hypersil ODS columns: 125 x 4.0 mm, 5 µm.

4. Manual syringe injector: With a 50 µL loop and a 50 µL syringe for injection (*see* **Note 6**).

2.3.2. HPLC analysis of phyllobilins

1. Solvent A: 50 mM KPi, pH 7.0 (*see* **Note 7**).

2. Solvent B: MeOH.

3. Solvent C: Milli-Q water.

4. C18 Hypersil ODS columns: 250 x 4.6 mm, 5 µm (*see* **Note 8**)

5. A coolable autosampler for injection (*see* **Note 9**).

2.3.3. HPLC system

A remote-controlled HPLC system interfaced with a photodiode array detector with an absorption range between 200 and 700 nm is required (*see* **Note 10**). The HPLC system should contain a pump able to produce a gradient of at least two solvents (*see* **Note 11**) and a thermostatted column

compartment set to 28° C (*see* **Note 12**). To avoid gas formation caused by mixing of solvents, an efficient degasser should be installed with the pump system (*see* **Note 13**).

2.4. Quantification

1. Chlorin standards : Several chlorin standards can be purchased from certified suppliers or are isolated from leaf extracts and purified by analytical HPLC as described here or according to published methods (for example, *(11, 12)*).

2. Calibration solutions for chlorins: Pure solutions of respective chlorins, prepared at defined concentrations (~ 2 mM) in acetone are used for calibrating HPLC peak absorptions (*see* **Note 14**).

3. *Cj*-NCC-1: Phyllobilin standards are not commercially available. *Cj*-NCC-1 is the major nonfluorescent chlorophyll catabolite from senescent leaves of *Cercidiphyllum japonicum*, a deciduous tree grown in many gardens and parks. *Cj*-NCC-1 can rather easily be isolated and purified by HPLC according to published methods *(13)* and can be used for NCC quantifications.

4. Calibration solution for *Cj*-NCC-1 (*see* **Note 15**): *Cj*-NCC-1 dissolved in H_2O is quantified spectrophotometrically at 314 nm ($\log \varepsilon_{314} = 4.23$; *(13)*) (*see* **Note 16**). Defined concentrations are used to calibrate HPLC peak absorptions and can be used for quantification of NCCs.

3. Methods

3.1. Extraction of chlorins (*see* **Notes 17** and **18**)

1. Harvest green or senescent plant material (*see* **Note 19**) and immediately flash-freeze in liquid nitrogen. Store at -80° C until use.

2. Grind plant material with mortar and pestle in liquid nitrogen to a fine powder (*see* **Note 20**).

3. Weigh 50-100 mg of plant material into a microcentrifuge tube (pre-cooled in liquid nitrogen) and keep the samples in liquid nitrogen until extraction (*see* **Note 21**).

4. For pigment extraction work on ice or at 4° C (*see* **Note 22**). Add 5-10 volumes of chlorin extraction buffer (v/w) to frozen plant material and vortex vigorously for around 10 sec until the plant material is thawed (*see* **Note 23**).

5. Incubate in the dark (*see* **Note 24**) at -20° C for 2-16 h until all pigments are extracted into the buffer (*see* **Note 25**).

6. Pellet plant extracts by centrifugation for 2 min at 16,000 x *g* and 4° C with a microcentrifuge. Transfer the supernatant into a new microcentrifuge tube and immediately flash-freeze and store in liquid nitrogen until HPLC analysis (*see* **Notes 26** and **27**).

3.2. Extraction of phyllobilins (*see* **Note 17**)

1. Harvest green or senescent plant material (*see* **Note 19**) and immediately flash-freeze in liquid nitrogen. Store at -80° C until use.

2. Grind plant material with mortar and pestle in liquid nitrogen to a fine powder (*see* **Note 20**).

3. Weigh 100-200 mg of plant material into a microcentrifuge tube (pre-cooled in liquid nitrogen) and keep the samples in liquid nitrogen until extraction (*see* **Note 21**).

4. Add 3 volumes of phyllobilin extraction buffer (v/w) and vortex vigorously for about 10-15 seconds until the plant material is thawed and uniformly mixed with the buffer.

5. Sonicate for 10 min in an ice-cooled sonication bath.

6. Centrifuge the mixture at 16,000 x *g* for 5 min with a microcentrifuge and then transfer the supernatant to a new 1.5 mL microcentrifuge tube.

7. Re-centrifuge the supernatant at 16,000 x *g* for 5 min with a microcentrifuge (*see* **Note 28**) and transfer 100-200 µL of the supernatant to a HPLC vial (*see* **Note 29**).

3.3. HPLC analysis and quantification of chlorins (*see* **Note 30**)

1. Remove a sample from liquid nitrogen just prior to injection and thaw it (*see* **Notes 6** and **31**). Inject a defined volume (e.g. 50 µL) into the HPLC system and run the following HPLC program.

2. HPLC program used for chlorin analysis (flow rate 1 ml min^{-1}):

Time [min]	**Solvent A**	**Solvent B**
0	100%	0%
15	0%	100%
25	0%	100%
28	100%	0%
32	100%	0%

3. Calibration: Inject standard solutions of respective chlorins and determine the retention times and absorption spectra. **Fig. 1** shows the spectra for different chlorins, and in **Table 1**, the retention times

are listed (*see* **Note 32**). Integrate peak areas of standard solutions with known concentrations (*see* **Note 14**) at 665 nm (*see* **Note 33**) and determine the relation between absorption and amount/concentration.

4. Identify the chlorins present in your sample via comparing the absorption spectra and retention times of peaks with standard solutions.

5. Integrate the peak areas at 665 nm and convert into amounts/concentrations using the factors calculated with standard solutions.

3.4. HPLC analysis of phyllobilins

1. Place samples in the precooled (7-10° C) autosampler of the HPLC system (*see* **Note 34**). Run the batch of samples by injecting defined volumes (e.g. 50 µL) with the following HPLC program.

2. HPLC program used for phyllobilin analysis (flow rate 1 ml min^{-1}):

Time [min]	**Solvent A**	**Solvent B**	**Solvent C**
0	80%	20%	0%
5	80%	20%	0%
35	40%	60%	0%
45	40%	60%	0%
47	40%	60%	0%
49	0%	100%	0%
54	0%	100%	0%
56	0%	60%	40%
58	80%	20%	0%
60	80%	20%	0%

3. Calibration: Inject defined amounts of *Cj*-NCC-1 standard solutions (*see* **Section 2.4.3**) into the HPLC. Integrate peak areas at 315 nm (*see* **Note 35**) and determine the relation between absorption and amount/concentration.

4. Identify the phyllobilins that are present in your sample via screening chromatograms at 254 nm (*see* **Note 35**) and search for peaks that exhibit UV/Vis absorption spectra typical for the different known types of phyllobilins (**Fig. 1**) (*see* **Note 36**).

5. For quantification of NCCs, integrate the peak areas at 320 nm and convert into amounts/ concentrations using the factors calculated with the *Cj*-NCC-1 standard.

6. Retention times of the major phyllobilins from senescent leaves of Arabidopsis *(5, 14)* are listed in Table 2 (*see* **Note 37**).

4. Notes

1. Chlorophyll is sensitive to acidic pH which causes artefactual pheophytin formation *(15)*.

2. Dilute the 10x KPi stock solution in milli-Q H_2O. Aqueous KPi buffers are prone to bacterial contaminations. Store aliquots of the 10x stock solution at -20° C. Once prepared, use the extraction buffer rather quickly.

3. For small sample amounts (100 mg or less), grinding is best done in a mixer mill (e.g. Retsch MM200, Haan, Germany). For this, place the plant tissue in 1.5 mL centrifuge tubes, add 5-10 glass beads (3 mm diameter) and freeze in liquid nitrogen. Place tubes in the Teflon holders provided with the mill (precooled in liquid nitrogen) and run at a frequency of 30 Hz for 3 x 30 sec with re-cooling in liquid nitrogen in-between.

4. Before use, degas solvents by sonicating in a sonication bath for 5 min to prevent gas formation within the HPLC system.

5. To avoid the effect of end volume changes when mixing an aqueous solution with an organic solvent, measure MeOH and the 1 M ammonium acetate solutions in separate measuring cylinders and subsequently combine them.

6. Autosamplers should not be employed for chlorin analysis, because of the instability of phytylated pigments even at 4° C, which is caused by chlorophyllases potentially still active in the extracts *(16)*. Instead, it is crucial for chlorin extracts to be kept in liquid nitrogen until injection into the HPLC.

7. Maintaining a pH of 7.0 is critical to avoid undesired FCC/DFCC to NCC/DNCC conversion during the HPLC run that may occur at low pH *(7, 17)*.

8. HPLC columns should be stored in MeOH:H_2O, 50:50 (v/v) to prevent precipitation of salts on the column.

9. Although an autosampler is not abolutely necessary, it allows running a batch of multiple samples without the need of manual injection. Consider, however, that phyllobilins are rather thermo-labile and light-sensitive, they should be cooled in the autosampler and be protected from light.

10. Availability of a fluorescence detector is advantageous for the detection of FCCs/DFCCs, which emit blue fluorescence at 450 nm when exited at 360 nm *(6)*.

11. If only two solvent channels are available, reduce the concentration of the KPi buffer to 10 mM for phyllobilin analysis. This is necessary, because high MeOH concentrations used during the HPLC run could cause precipitation of phosphate salts.

12. A temperature-controlled column is recommended for phyllobilin separation to guarantee uniform retention times. This is however, less critical for separation of chlorins.

13. Gas formation is particularly critical during phyllobilin analysis when mixing the KPi buffer (solvent A) with MeOH (solvent B). If no degasser is available, premix the KPi buffer with MeOH to the start conditions (i.e. 20% MeOH, when using the gradient provided in **Section 3.4.2**), desgas by sonication and adjust the HPLC gradient accordingly.

14. If the concentrations of chlorin standards is unknown, e.g. because of manual extraction and preparation of pigments (*see* **Section 2.4.1**), the concentration can be determined spectrophotometrically prior to HPLC injection. For calculating concentrations use the following equations valid for pigments in 80% acetone *(18, 19)* (A = absorption at indicated wavelength):

chlorophyll *a* or chlorophyllide *a* [µg ml^{-1}] = 11.63 A_{665} – 2.39 A_{649}

chlorophyll *b* or chlorophyllide *b* [µg ml^{-1}] = 20.11 A_{649} – 5.18 A_{665}

pheophytin *a* or pheophorbide *a* [µg ml^{-1}] = 22.42 A_{665} – 6.81 A_{653}

pheophytin *a* or pheophorbide *a* [µg ml^{-1}] = 40.17 A_{653} – 18.58 A_{665}

15. Due to the commercial unavailability of phyllobilin standards and the limited spectral data available for the different classes of phyllobilins, relatively accurate quantification is so far only possible for NCCs.

16. Rather similar log ε values have been determined for NCCs from different plant species (for example, *(20, 21, 22)*), allowing the approximate quantification of (unknown) NCCs from any plant sample using *Cj*-NCC-1 as standard.

17.With the method presented, chlorins or phyllobilins have been analyzed in leaves and fruits of different plant species such as Arabidopsis and tomato *(23, 24)*. If other organs or plant species are analyzed, the method might require adaptations, in particular concerning the ratio of tissue amount to extraction buffer volume and the time of extraction.

18.An extensive comparison of different methods for chlorophyll extraction, including a sub-zero temperature method using acetone similar to the one described here, has recently been published *(16)*.

19.Senescence induction in leaves (e.g. Arabidopsis) can be performed by incubating detached leaves in the dark. Leaves are cut and incubated on wet filter paper in a closed container at room temperature in the dark. Induction of senescence can be detected after 3-5 days. Alternatively, leaves can be covered with aluminum foil while being attached to the plant. If induction of leaf senescence is applied to other plant species, the procedure might require adaptations.

20.Work fast and make sure that the plant material does not thaw during the procedure.

21.To avoid thawing of the tissue during transferring to the microcentrifuge tube, dip the microcentrifuge tube in liquid nitrogen and use it as a shuffle to transfer the desired amount of tissue. Immediately, weigh the sampled tissue using a fine balance that has been tared with an empty microcentrifuge tube.

22.Chlorophyllases are ubiquitously present in plant tissues and might get activated during the process of pigment extraction. Therefore it is very important to work quickly and in a cold environment, in order to prevent artefactual chlorophyllide and/or pheophorbide formation *(16, 23)*.

23.The required amount of buffer depends on chlorophyll concentration. Five volumes of buffer are generally sufficient for leaves of Arabidopsis. For tomato or other species that contain high amounts of chlorophyll, the buffer volume may need to be increased.

24.Make sure samples are not exposed to light to prevent bleaching of the pigments.

25.Extraction time depends on the plant sample. For green or senescent Arabidopsis leaves, 2 h of incubation is normally sufficient. For other plant tissues that are more fibrous and/or contain high chlorophyll levels (such as tomato or barley leaves), extraction can be done overnight. Alternatively, 2-3 rounds of extraction each followed by a centrifugation step can be done with subsequent pooling of extracted fractions or extracts may be sonicated for a few minutes in an ice-cooled sonication bath.

26.Transfer as much supernatant as possible, but ensure that no solids are transferred.

27.Only when pellets are colorless, all chlorins have been extracted into the acetone phase. If the pellet still appears green, multiple rounds of extraction can be performed (*see* also **Note 25**).

28.Twice centrifugation ensures that samples are free of solid particles. This may particularly be necessary with leaf samples from e.g. Poaceae species, like barley, which are rather fibrous.

29.If HPLC analysis will be performed at a later time point, prepared HPLC vials can be frozen in liquid nitrogen.

30.The chlorin extraction procedure described in **Section 3.1** simultaneously extracts carotenoids from plant tissues. Thus, HPLC analysis of these extracts allows the simultaneous analysis of carotenoids. Methods for carotenoid analysis and quantification have been published *(25)*.

31.Frozen acetone develops high pressure while thawing. To avoid possible explosion of the microcentrifuge tube, release pressure by opening the microcentrifuge lid.

32.These values were obtained with the following HPLC system: column: C18 Hypersil ODS column (125 x 4.0 mm, 5 µm) (MZ Analysentechnik), pump: Gynkotek High Precision Pump Model 480 (Thermo Fisher Scientific); photodiode array detector: 206 PHD (365-700 nm; Linear); software: ChromQuest version 2.51 (Thermo Fisher Scientific). For other HPLC system, retention times may vary.

33.Although peak maxima differ between different chlorins (**Fig. 1**), 665 nm is an ideal wavelength for simultaneous quantification of all chlorins.

34.If sample-containing HPLC vials had been stored in liquid nitrogen, make sure no air bubbles are trapped in the conical tip of the HPLC vials.

35.254 nm is best suited for detection of all types of phyllobilin, while e.g. DNCCs do only weakly absorb at 315 nm *(5)*, the wavelength suitable for quantification of NCCs.

36.Peak resolution and baseline settings are important parameters for accurately quantifying NCC peaks. Senescent leaves of different plant species tend to accumulate phyllobilin-unrelated compounds that absorb in the UV range and may co-elute with phyllobilins, and, thus, may interfere with phyllobilin identification and quantification.

37.These values were obtained with the following Dionex HPLC system (Thermo Fisher Scientific): column: C18 Hypersil ODS column (250 x 4.6 mm, 5 µm); autosampler: AS-100; column compartment: TCC-100 (set to 28°C); detectors: PA-100 photodiode array detector (200-700 nm) and RF2000 fluorescence detector (excitation at 360 nm, emission at 450 nm); software: Chromeleon 6.8 chromatography data system. For other HPLC system, retention times may vary.

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation and by CropLife, an EU Marie-Curie Initial Training Network.

References

1. Hörtensteiner S., Kräutler B. (2011) Chlorophyll breakdown in higher plants. *Biochim. Biophys. Acta* **1807**,977-988
2. Christ B., Hörtensteiner S. (2014) Mechanism and significance of chlorophyll breakdown. *J. Plant Growth Regul.* **33**,4-20
3. Sakuraba Y., Schelbert S., Park S.-Y. et al. (2012) STAY-GREEN and chlorophyll catabolic enzymes interact at light-harvesting complex II for chlorophyll detoxification during leaf senescence in *Arabidopsis*. *Plant Cell* **24**,507-518
4. Christ B., Schelbert S., Aubry S. et al. (2012) MES16, a member of the methylesterase protein family, specifically demethylates fluorescent chlorophyll catabolites during chlorophyll breakdown in Arabidopsis. *Plant Physiol.* **158**,628-641
5. Christ B., Süssenbacher I., Moser S. et al. (2013) Cytochrome P450 CYP89A9 is involved in the formation of major chlorophyll catabolites during leaf senescence in *Arabidopsis*. *Plant Cell* **25**,1868-1880
6. Kräutler B. (2014) Phyllobilins - the abundant bilin-type tetrapyrrolic catabolites of the green plant pigment chlorophyll. *Chem. Soc. Rev.* **43,**6227-6238
7. Oberhuber M., Berghold J., Breuker K. et al. (2003) Breakdown of chlorophyll: a nonenzymatic reaction accounts for the formation of the colorless "nonfluorescent" chlorophyll catabolites. *Proc. Natl. Acad. Sci. USA* **100**,6910-6915
8. Moser S., Müller T., Ebert M.O. et al. (2008) Blue luminescence of ripening bananas. *Angew. Chem. Int. Ed.* **47**,8954-8957
9. Kräutler B., Banala S., Moser S. et al. (2010) A novel blue fluorescent chlorophyll catabolite accumulates in senescent leaves of the peace lily and indicates a divergent path of chlorophyll breakdown. *FEBS Lett.* **584**,4215-4221
10. Ulrich M., Moser S., Müller T. et al. (2011) How the colourless 'nonfluorescent' chlorophyll catabolites rust. *Chem. Eur. J.* **17**,2330-2334
11. Shioi Y., Fukae R., Sasa T. (1983) Chlorophyll analysis by high-performance liquid chromatography. *Biochim. Biophys. Acta* **722**,72-79
12. Perkins H.J., Roberts D.W.A. (1962) Purification of chlorophylls, pheophytins and pheophorbides for specific activity determinations. *Biochem. Biophys. Acta* **58**,486-498
13. Curty C., Engel N. (1996) Detection, isolation and structure elucidation of a chlorophyll *a* catabolite from autumnal senescent leaves of *Cercidiphyllum japonicum*. *Phytochemistry* **42**,1531-1536

14. Pružinská A., Tanner G., Aubry S. et al. (2005) Chlorophyll breakdown in senescent Arabidopsis leaves: characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction. *Plant Physiol.* **139**,52-63
15. Mazaki H., Watanabe T., Takahashi T. et al. (1992) Pheophytination of eight chlorophyll derivatives in aqueous acetone. *Bull. Chem. Soc. Jpn.* **65**,3212-3214
16. Hu X.Y., Tanaka A., Tanaka R. (2013) Simple extraction methods that prevent the artifactual conversion of chlorophyll to chlorophyllide during pigment isolation from leaf samples. *Plant Methods* **9**,19
17. Oberhuber M., Berghold J., Kräutler B. (2008) Chlorophyll breakdown by a biomimetic route. *Angew. Chem. Int. Ed.* **47**,3057-3061
18. Lichtenthaler H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Meth. Enzymol.* **148**,350-382
19. Strain H.H., Cope B.T., Svec W.A. (1971) Analytical procedures for the isolation, identification, estimation and investigation of the chlorophylls. *Methods Enzymol.* **23**,452-476
20. Kräutler B., Jaun B., Bortlik K.-H. et al. (1991) On the enigma of chlorophyll degradation: the constitution of a secoporphinoid catabolite. *Angew. Chem. Int. Ed. Engl.* **30**,1315-1318
21. Berghold J., Eichmüller C., Hörtensteiner S. et al. (2004) Chlorophyll breakdown in tobacco: on the structure of two nonfluorescent chlorophyll catabolites. *Chem. Biodivers.* **1**,657-668
22. Scherl M., Müller T., Kräutler B. (2012) Chlorophyll catabolites in senescent leaves of the lime tree (*Tilia cordata*). *Chem. Biodivers.* **9**,2605-2617
23. Schenk N., Schelbert S., Kanwischer M. et al. (2007) The chlorophyllases AtCLH1 and AtCLH2 are not essential for senescence-related chlorophyll breakdown in *Arabidopsis thaliana*. *FEBS Lett.* **581**,5517-5525
24. Guyer L., Schelbert Hofstetter S., Christ B. et al. (2014) Different mechanisms are responsible for chlorophyll dephytylation during fruit ripening and leaf senescence in tomato. *Plant Physiol.* **166,**44-56
25. Guzman I., Yousef G.G., Brown A.F. (2012) Simultaneous extraction and quantitation of carotenoids, chlorophylls, and tocopherols in Brassica vegetables. *J. Agric. Food Chem.* **60**,7238-7244

Figure captions

Fig. 1. Constitutional formulas and UV/Vis absorption spectra of chlorophyll-derived chlorins and phyllobilins occurring during chlorophyll breakdown.

Tables

Table 1. Approximate retention times of chlorins using the HPLC system outlined in **Note 32**.

Pigment	Retention time [min]
Chlorophyllide *b*	1.5
Chlorophyllide *a*	3
Pheophorbide *b*	7
Pheophorbide *a*	9

Chlorophyll *b*	16
Chlorophyll *a*	18
Pheophytin *b*	21
Pheophytin *a*	23

Table 2. Approximate retention times of the major *Arabidopsis* phyllobilins using the HPLC system outlined in **Note 37**.

Phyllobilin	Retention time [min]
At-DNCC-1	21.5
At-NCC-1	23.7
At-DNCC-2	24.7
At-NCC-2	26.4
At-DNCC-3	27.3
At-NCC-3	27.8
At-NCC-4	29.1
At-DNCC-4	29.2
At-DNCC-5	30.5
At-NCC-5	36.2